REMARKS

Applicant acknowledges the supplemental election made on December 21, 2004 of group I, drawn to the 200 kDa antigen specific monoclonal antibodies and hybridomas, to prosecute in the instant application.

New claims 50-57 are pending in this application. Claims 1-49 have been cancelled without prejudice. Applicant reserves the right to prosecute the subject matter of any canceled claim in one or more continuation, continuation-in-part, or divisional applications. Support for the new claims may be found in the specification. Specifically, example of support for claims 50 and 56 can be found on page 7, lines 5-11, page 8, lines 19-24, page 20, lines 6-10, and originally filed claims 8-9, 15-17 of the instant specification. Support for claim 52 can be found on page 19, line 25 to page 20, line 1 and originally filed claim 12 of the instant specification. Support for claim 53 can be found on page 10, line 27 to page 11, line 1, page 40, lines 3-6, and originally filed claim 37 of the instant specification. Support for claims 54-55 can be found on page 35, line 19 to page 37, line 12 and originally filed claims 38-39 of the instant specification. Support for claims 51 and 57 can be found on page 7, line 12 to page 8, line 4, page 19, lines 20-25, page 20, lines 6-20, page 40, lines 10-24, and originally filed claims 8-9, 14, 18 of the instant specification.

Thus, the new claims are fully supported by the instant specification and no new matter has been introduced.

Claim Objections

Claims 1-12, 14-21, 35-39, and 49 were objected to for being drawn to multiple inventions. Applicant contends that new claims 50-58 are directed to the one invention elected on December 21, 2004 - - namely the 200 kDa antigen specific monoclonal antibodies and hybridomas producing the same. Accordingly, Applicant respectfully requests withdrawal of this objection.

Rejections Under §112

Claims 2, 7-12, 15-19, 35-39, and 49 were rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the enablement requirement. The Examiner points out that the claims recite specific monoclonal antibodies and hybridoma cell lines. However, the Examiner is unclear as to whether the recited monoclonal antibodies and hybridoma cell lines are readily available to the public, *e.g.*, by a deposit made under the terms of the Budapest Treaty.

Applicant submits herewith a Receipt in the Case of an Original Deposit Issued Pursuant to Rule 7.3 and Viability Statement Issued Pursuant to Rule 10.2 ("the Receipt", attached hereto as Exhibit 1) from the American Type Culture Collection, the depository. The Receipt shows that the hybridoma cell lines recited in the claims were deposited under the terms of the Budapest Treaty. Additionally, the specification has been amended to include the required identifying information pursuant to 37 C.F.R §1.809(d). Accordingly, Applicant respectfully requests withdrawal of this rejection.

Claims 35-37 and 49 were rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the enablement requirement. The Examiner alleges that the specification does not disclose how to use the monoclonal antibodies of the invention as a

pharmaceutical and thus undue experimentation would be required to practice the invention. Applicant respectfully disagrees.

New claim 53 is directed to pharmaceutical compositions comprising the monoclonal antibodies of the invention or antigen binding fragment thereof and a pharmaceutically acceptable carrier, excipient, or diluent. While acknowledging that the specification teaches that several monoclonal antibodies were made using small cell lung cancer (SCLC) cells that recognize a 200 kDa antigen, the Examiner contends that the specification fails to provide guidance that would enable the skilled artisan on how to use pharmaceutical compositions comprising the monoclonal antibodies. The Examiner alleges that, although antibody internalization is crucial for the use of drugs or immunotoxins in immunoadaptive therapy, the monoclonal antibodies of the invention have not been shown to be internalized *in vivo*. Furthermore, the Examiner alleges that cancer cells *in vitro* behave differently than cancer cells *in vivo*, thus, based on the cell culture data present in the instant specification, it could not be predicted if the monoclonal antibodies of the invention would "work" *in vivo*. The Examiner relies on Krueger et al., 2003, Cancer Immunol. Immunother. 52:367 ("Krueger"); Freshney, 1983, Culture of Animal Cells, A Manual of Basic Technique ("Freshney"); and Dermer, 1994, BioTechnology 12:230 ("Dermer") to support the allegations.

By emphasizing the distinction between *in vitro* and *in vivo* uses of the claimed pharmaceutical compositions, the Examiner predicates her position on the existence of a heightened how-to-use standard for pharmaceutical compositions. The case law does not support the Examiner's position. Nor has the Examiner provided any support for this contention.

The case law clearly distinguishes between claims drawn to a compound and claims drawn to the therapeutic use of the compound, the latter requiring a greater burden. *See, e.g.*, In re Bundy, 642 F.2d 430, 209 USPQ 48 (CCPA 1981). However, the law does not distinguish between claims drawn to a compound and claims drawn to a pharmaceutical composition comprising the compound. The only relevant question is whether the claim recites a therapeutic use. The present claims do not recite a therapeutic use.

The Examiner has focused seemingly exclusively on the use of the claimed pharmaceutical compositions for administration to an individual for SCLC treatment. The references cited *supra* discuss treatment of cancer, especially those treatments using drug- or immunotoxin-conjuated antibodies, and the potential issues that arise when extrapolating from *in vitro* data to treatment efficacy *in vivo*. However, Applicant would like to point out that the specification discloses a number of uses for pharmaceutical compositions comprising monoclonal antibodies of the invention in addition to methods of SCLC treatment. For example, the monoclonal antibodies of the invention can be administered to an individual in a pharmaceutical composition to localize and/or image SCLC cells *in vivo* (see, *e.g.*, page 10, lines 10-17 and page 28, lines 3-9 of the instant specification). Additionally, the specification clearly teaches the use of the claimed pharmaceutical compositions for immunizing animals to produce anti-ideotypic antibodies[1] (see, *e.g.*, page 9, line 26 to page 10, line 2 of the instant specification).

When a claim is drawn to a composition but does not recite a specific use of the composition, any objective disclosed in the specification and enabled is sufficient to meet the requirement of the "how-to-use" prong of 35 U.S.C. § 112, first paragraph. Raytheon Company vs. Roper Corporation, 724 F.2d 951, 958 (Fed. Cir. 1983). MPEP 2164.01(c)

[1] Anti-ideotypic antibodies are antibodies that recognize the hypervariable (antigen recognition) region of another antibody.

precludes a rejection for nonenablement based on how to use when any enabled use is presented that would reasonably correlate with the entire scope of that claim stating in part:

> [W]hen a compound or composition claim is not limited by a recited use, any enabled use that would reasonably correlate with the entire scope of that claim is sufficient to preclude a rejection for nonenablement based on how to use. If multiple uses for claimed compounds or compositions are disclosed in the application, then an enablement rejection must include an explanation, sufficiently supported by the evidence, why the specification fails to enable each disclosed use. In other words, if any use is enabled when multiple uses are disclosed, the application is enabling for the claimed invention.

Applicant respectfully submits that the Examiner has failed to present a reasoned basis for her position that the *in vitro* experiments disclosed in the specification do not provide adequate expectation of success in achieving *any* constructive results in administering a pharmaceutical composition comprising the monoclonal antibodies of the invention. Even assuming *en arguendo* that the *in vitro* data does not enable SCLC treatment as the Examiner contends, other disclosed uses for pharmaceutical compositions are enabled. For example, the *in vitro* data clearly shows that the monoclonal antibodies of the invention can bind to the 200 kDa surface antigen on SCLC cells from immortal cell lines (see, *e.g.*, Examples 2 and 3 of the instant specification) as well as primary tissue samples taken from patients (see, *e.g.*, Example 4 of the instant specification). The primary tissue samples clearly had not undergone the "evolutionary step" of "adapting to the immortal life in culture" as Dermer warns or had suffered the "dissociation from a three-dimensional geometry" as described by Freshney. Additionally, in order to bind to and localize SCLC cells *in vivo*, the antibody does not have to become internalized as Krueger states in connection with delivery of a toxin to a cell for drug therapy. Furthermore, production of anti-ideotypic antibodies only require injection of the monoclonal antibody into an immunocompetent animal. The

presence or absence of SCLC cells and whether they are primary cells or an immortal cell line is irrelevant. The rejection of the claims based on the Examiner's opinion, without additional evidence, is impermissible. *See, e.g.*, In re Zeidler, 682 F.2d 961, 967 (CCPA 1982). Thus, the Examiner is invited to provide additional evidence as to why the only use of a pharmaceutical composition comprising the antibodies of the invention is for SCLC treatment if she is in disagreement with the foregoing assertion.

The instant composition claims are not limited by any specific use. Thus, any enabled use would be sufficient to support the claims. Accordingly, the specification teaches the use of pharmaceutical compositions for the identification and localization of SCLC cells in vivo and the production of anti-ideotypic antibodies. Either use is a sufficient utility to satisfy the how-to-use requirement of 35 U.S.C. § 112, first paragraph.

Claims 1, 3, 8, 10, 11, 14, 18, 35-39, and 49 were rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the written description requirement. The Examiner alleges that the instant specification does not adequately describe the 200 kDa antigen and as such has not adequately described the genus of monoclonal antibodies that bind to the antigen. Although Applicant respectfully disagrees, in order to further prosecution, the presently pending claims encompass a smaller genus of monoclonal antibodies.

New independent claims 50 and 56 encompass monoclonal antibodies and hybridomas that have been deposited with the ATCC - - namely monoclonal antibodies MoAb 51.2, MoAb 37.14, MoAb 109.12, or MoAb 26.1. The Examiner has acknowledged that these antibodies and hybridomas have been adequately described (see page 10, lines 17-18 of the Office Action mailed December 28, 2004).

New independent claims 51 and 57 encompass monoclonal antibodies that competitively inhibit any one of monoclonal antibodies MoAb 51.2, MoAb 37.14, MoAb 109.12, or MoAb 26.1 and hybridomas which produce such competitor antibodies. Competitive inhibition is the assaying of pairs of monoclonal antibodies for the ability to simultaneously bind to an antigen. Monoclonal antibodies directed against separate epitopes will bind independently of each other, whereas monoclonal antibodies directed against the same epitope or epitopes in close physical proximity (*e.g.*, overlapping) will interfere with each other's binding. Thus, antibodies can be assayed for the ability to inhibit binding of any of monoclonal antibodies MoAb 51.2, MoAb 37.14, MoAb 109.12, or MoAb 26.1 to the antigen which they recognize. Those antibodies that can inhibit the binding of any of monoclonal antibodies MoAb 51.2, MoAb 37.14, MoAb 109.12, or MoAb 26.1 are encompassed by the pending claims. Thus, the claims no longer encompass any antibody that binds to any epitope on the 200kDa antigen.

While the instant specification does not specifically recite the phase "competitively inhibit," it does disclose, for example, that the invention encompasses those monoclonal antibodies that are "...capable of binding to the same antigenic determinant on SCLC as do the monoclonal antibodies described herein..." (page 7, lines 12-14 of the instant specification). According to applicable case law, "ipsis verbis disclosure is not necessary to satisfy the written description requirement of section 112. Instead, the disclosure need only reasonably convey to persons skilled in the art that the inventor had possession of the subject matter in question." Fujikawa v. Wattanasin, 93 F.3d 1559, 39 USPQ 2d 1895, 1904 (Fed. Cir. 1996).

In this instance, although the exact phrase used in the amended claims was not used in the specification, Applicant submits that the recitation does not add any subject

matter that which the specification does not already disclose or was known in the art at the time of filing. Information which is well known in the art need not be described in detail in the specification. Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1379-80 (Fed. Cir. 1986).

Competitive inhibition assays were used in the art to determine epitope specificity. There are many examples in the art prior to the time of filing the instant application that use competitive inhibition assays to determine if multiple antibodies bound to the same or different epitopes on an antigen. For example, two monoclonal antibodies directed to the same polypeptide were determined to bind the same epitope using competitive inhibition assays in Sher et al. (1984, J. Immunol. 133:338; attached hereto as Exhibit 2). A decrease in the amount of labeled antibody binding to cells expressing its antigen was observed in the presence of the other (unlabelled) antibody causing the authors to conclude that the two antibodies "identify the same or overlapping epitopes" (see page 339, col. 2, the first two paragraphs of the Results section of Sher). Additionally, five monoclonal antibodies raised to a synthetic peptide were all found to bind the same epitope using competitive inhibition assays in Katai et al. (1991, Molec. Cell. Biochem. 101:73-81; attached hereto as Exhibit 3). The synthetic peptide was coated on an ELISA dish and incubated with a labeled monoclonal antibody. An excess of unlabelled monoclonal antibody was added to the dish and a decrease in the amount bound, labeled antibody was interpreted as evidence that the two antibodies bound to the same epitope (see page 76-78, section titled "Epitope specificity of monoclonal antibodies" and Figure 2 of Katai). Thus, the recitation in the specification of antibodies that bind the same epitope on SCLC as do monoclonal antibodies MoAb 51.2, MoAb 37.14, MoAb 109.12, or MoAb 26.1 could direct one skilled in the art to perform competitive inhibition assays to determine such antibodies.

For the above reasons, Applicant respectfully requests reconsideration and withdrawal of the rejections under §112.

Rejection Under §102

Claims 1, 3, 8, 10, 11, 14, 18, 35-39, and 49 were rejected under 35 U.S.C. §102(b) as anticipated by Rose et al., 1994, Hybridoma 13:221-227 ("Rose"). The Examiner alleges that the claims encompass the CR101 monoclonal antibody described in Rose and as such CR101 would be anticipatory. Applicant respectfully disagrees.

Applicant would like to point out that the claims have been amended to encompass monoclonal antibodies MoAb 51.2, MoAb 37.14, MoAb 109.12, MoAb 26.1, and competitive inhibitors of the same.

Furthermore, Applicant would like to highlight a difference between the antigens bound by the CR101 monoclonal antibody of Rose and the claimed antibodies. According to Rose, the antigen recognized by CR101 is a highly glycosolated cell surface antigen associated with SCLC that resolves as two proteins of 94 kDa and 115 kDa by SDS-PAGE after enzymatic removal of the associated sugars (see, *e.g.*, the Abstract, the last paragraph on page 221, Table 1, and Figure 6 of Rose). In contrast, when the antigen recognized by the antibodies of the present invention is deglycosolated, a single protein of slightly less than 98 kDa is resolved by SDS-PAGE. This data is present in Krueger, a reference co-authored by Applicant reporting data obtained using monoclonal antibodies MoAb 51.2, MoAb 37.14, MoAb 109.12, MoAb 26.1. The data pertaining to the deglycosolation experiment described *supra* was conducted with monoclonal antibody MoAb 51.2 (see, *e.g.*, Figure 2 and the last full paragraph in the first column on page 371 of Kruger).

"A rejection for anticipation under section 102 requires that each and every limitation of the claimed invention be disclosed in a single prior art reference." In re Paulsen,

30 F.3d 1475, 31 USPQ2d 1671 (Fed. Cir. 1994). CR101 of Rose is not encompassed by the present claims and thus does not meet each and every claim limitation.

For the above reasons, Applicant respectfully requests reconsideration and withdrawal of the rejection under §102.

Rejection Under §103

Claims 1 and 12 were rejected under 35 U.S.C. §103(a) as being unpatentable over Rose in view of Ward, 1992, Antibody Engineering, WH Freeman and Company, Car and Borrenbaek, ed. Pages 122-123 ("Ward"). The Examiner alleges that because the monoclonal antibodies of the invention are taught by Rose, Fab fragments of the antibodies are obvious to make in view of Ward. Applicant respectfully disagrees.

A finding of obviousness under 35 U.S.C. § 103 requires a determination of the scope and the content of the prior art, the differences between the invention and the prior art, the level of the ordinary skill in the art, and whether the differences are such that the claimed subject matter as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. Graham v. Deere, 383 U.S. 1 (1966). The relevant inquiry is whether the prior art suggests the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful. In re O'Farrell, 853 F.2d 894, 902-4 (Fed. Cir. 1988); In re Vaeck, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed. Cir. 1991). Both the suggestion of the claimed invention and the expectation of success must be in the prior art, not in the disclosure of the claimed invention. In re Dow Chemical Co., 5 U.S.P.Q. 2d 1529 (Fed. Cir. 1988).

Applicant contends that the antibodies encompassed by the present claims are not reported by Rose for the reasons discussed *supra*. As such, Fab fragments of the novel antibodies of the invention are not obvious. Applicant acknowledges that Fab fragments in

general were known at the time of the invention, however, Fab fragments of the particular antibodies of the present invention were not known.

For the above reasons, Applicant respectfully requests reconsideration and withdrawal of the rejection under §103.

CONCLUSION

It is believed that the elected claims are in condition for allowance. Early and favorable action by the Examiner is earnestly requested.

AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. 13-4500, Order No. 3828-4001US1.

Respectfully submitted,
MORGAN & FINNEGAN, L.L.P.

Dated: June 28, 2005

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Vacold, LLC
Attn: Cohava Gelber, Ph.D.
765 Old Saw Mill River Road
Tarrytown, NY 10591

Deposited on Behalf of: Vacold LLC

Identification Reference by Depositor:	**Patent Deposit Designation**
Mouse Hybridoma (Sp 2/0): IMM010.109.12	PTA-2357
Mouse Hybridoma (Sp 2/0): IMM010.37.14	PTA-2358
Mouse Hybridoma (Sp 2/0): IMM010.26.1	PTA-2359
Mouse Hybridoma (Sp 2/0): IMM010.51.2	PTA-2360

The deposits were accompanied by: __ a scientific description , a proposed taxonomic description indicated above. The deposits were received August 10, 2000 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested September 7, 2000. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Tanya Nunnally, Patent Specialist, Patent Depository

Date: January 31, 2001

cc: David E. Brook, Esq.

Exhibit 1
Application Serial No. 10/015,728
Docket No. 3828-4001 US1

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TWO Ia.17-SPECIFIC MONOCLONAL ANTIBODIES DETECT THE SAME EPITOPE BUT DO NOT SHARE IDIOTYPE[1]

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The clonal diversity of anti-Ia antibodies was studied by using heterologous anti-idiotypic reagents generated against two Ia.17-specific monoclonal antibodies (Mab). The Mab 10-2.16 and 10-3.6 are specific for the Ia.17 public specificity expressed on I-A molecules of the k, s, f, u, r, and j haplotypes. Competitive inhibition experiments demonstrated that 10-2.16 and 10-3.6 inhibited the binding of each other to Ia.17-positive cell targets, indicating that they detected the same or overlapping epitope(s). Identical inhibition patterns of ^{125}I-10-2.16 binding by 10-3.6 by using Ia.17-positive target cells of four different strains indicated that inhibition was not due to steric hindrance from binding to spatially related epitopes. Rabbit anti-10-2.16 serum detected both site-specific and framework-specific 10-2.16 idiotypic determinants not shared by 10-3.6. Conversely, rabbit anti-10-3.6 serum detected 10-3.6 idiotypic determinants not shared by 10-2.16. C3H/J (I-A^k) anti-10-2.16 serum detected only 10-2.16 framework-specific idiotypes, suggesting that 10-2.16 site-specific determinants represent cellular determinants for syngeneic Ia antigens. Furthermore, anti-Ia.17 immune serum from three of 10 mice with the Igh-C^b allotype expressed the 10-2.16 framework-specific idiotype. We have demonstrated that two anti-Ia.17 Mab, 10-2.16 and 10-3.6, lack a shared idiotype, even though they detect a similar epitope. This observation and the variable expression of 10-2.16 idiotypic determinants in immune sera indicate that the B cell response to the Ia.17 epitope detected by 10-2.16 and 10-3.6 is diverse.

The I region gene products (Ia antigens) of the major histocompatibility complex (MHC)[4] play a critical role in the regulation of immune responses, and as membrane-bound glycoproteins, Ia antigens elicit significant humoral and cellular alloreactive responses (1, 2). Although it can be demonstrated that Ia antigens restrict syngeneic immune responses at the level of macrophage-T cell interactions and that the restriction operates during induction and regulation of immune responses, the mechanism of this Ia antigen-mediated control remains unknown (3–6). One approach to investigating Ia molecule regulation in the immune response involves defining the structure and specificity of T and B cell receptors for Ia determinants.

Ia antigen specificities and their regulatory functions have been defined by conventional anti-Ia alloantibodies, although the polyclonal nature of these conventional probes has made experiments difficult to reproduce and interpret (7–9). The recent development of anti-Ia monoclonal antibodies (Mab) has overcome many of these difficulties by providing better definition of the specificity and diversity of immunoglobulin receptors for Ia antigens. Anti-Ia Mab have identified previously unrecognized Ia epitopes as well as multiple epitopes corresponding to Ia specificities previously defined by heterogeneous alloantisera (10–14). For example, a panel of anti-I-E^k Mab have identified three separate clusters of epitopes that correlate to the Ia.7 specificity (14).

Furthermore, characterization of the idiotypy of anti-Ia Mab by xenogeneic anti-idiotypic sera has provided insight into the clonal diversity of Ia-specific B cell responses. Because cross-reactive idiotype (CRI) has been observed for anti-Ia Mab specific for several Ia specificities, it would appear that a limited idiotypic repetoire is available for Ia-specific antibodies (15–20). In this report, however, two Ia.17-specific Mab, 10-2.16 and 10-3.6, were shown to detect the same Ia epitope yet were found not to share idiotype. In addition, the 10-2.16 idiotype was found to have variable expression in anti-Ia.17 immune sera. These findings suggest that the clonal diversity of Ia-specific B cell receptors is potentially great.

MATERIALS AND METHODS

Animals. All mice were bred in our colony at the University of Michigan. Original breeders were obtained from The Jackson Laboratory, Bar Harbor, ME or from Dr. Donald Shreffler, Washington University, St. Louis, MO. Rabbits were purchased from Charles River Laboratories, Wilmington, DE.

Monoclonal antibodies (Mab). The properties of Mab used are summarized in Table I. Clones 10-2.16, 10-3.6, 11-5.2, and 14-4-4S were obtained through the Salk Institute, San Diego, CA. Clone 17-227 was obtained through American Type Culture Collection, Rockville, MD, and LPC-1 was obtained from the National Cancer Institute (NCI contract N01-CB-25584). Clone 103.1C9 was produced in the laboratory of Dr. Latham Claflin at the University of Michigan, and MOPC-21 was a gift from Dr. Michael Potter, (NIH, Bethesda, MD). All antibodies were purified from culture supernatants or ascites by affinity chromatography on protein A coupled to Sepharose 4B and were eluted with 0.1 M glycine-HCl, pH 2.2, buffer (21).

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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[4] Abbreviations used in this paper: MHC, major histocompatibility complex; Mab, monoclonal antibody; CRI, cross-reactive idiotype; BNHS, biotynl-*N*-hydroxy-succinimide; IDI, individual idiotype.

TABLE I

Characteristics of Mab

Antibody	Origin	IgG Isotype	Igh-C Allotype	Specificity
10-2.16	CWB	2b,κ[a]	b	Ia.17
10-3.6	CWB	2a,κ[a]	b	Ia.17
MOPC-21	BALB/c	1,κ	a	—
11-5.2	CWB	2a,κ[a]	b	Ia.2
LPC-1	BALB/c	2a,κ	a	—
103.1C9	PL/J	2b,κ	j	PC
14-4-4S	C3H.SW	2a,κ	j	Ia.7

[a] Express additional MOPC-21 light chain.

Production of xenogeneic anti-idiotype antibodies. Adult rabbits were immunized by intradermal injection with 400 μg of Mab in 200 μl saline emulsified with an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI). The rabbits were boosted 3 wk later with the same amount of protein in incomplete Freund's adjuvant. Bleedings were obtained weekly beginning 17 days after the initial immunization. Serum from each bleeding was tested for anti-mouse immunoglobulin activity against normal mouse serum by a capillary precipitation test.

Purification of xenogeneic anti-idiotypic antibody. The rabbit anti-idiotypic sera were precipitated twice in 40% saturated ammonium sulfate. The γ-globulin fraction of serum was extensively absorbed by sequential passage over three affinity columns. Sera were initially absorbed on a column comprised of five murine myeloma proteins representing five immunoglobulin isotypes coupled to Sepharose 4B. This absorption was followed by passage over a column of 103.1C9 (IgG2b) coupled to Sepharose 4B, and finally C57BL/6 immunoglobulin coupled to Sepharose 4B. Adsorption and subsequent elution of rabbit anti-10-2.16 antibodies to 10-2.16 coupled to Sepharose 4B did not increase specificity but did decrease protein yield. Anti-idiotypic specificity was assessed by competitive inhibition radioimmunoassay (RIA) (described below).

Production and purification of allogeneic anti-idiotypic antibodies. Allogeneic anti-10-2.16 serum was prepared according to the method described by Tung *et al.* (22). Briefly, 200 μl of an emulsion of CFA and 10-2.16 (25 mg/ml) at a 9:1 ratio (v/v) were injected i.p. into C3H/J mice at weekly intervals. The γ-globulin fraction of ascites was obtained by precipitation twice in 40% saturated ammonium sulfate.

Alloimmunization of mice. Alloantisera containing the Ia.17 specificity were prepared in 10 BALB/c mice (Igh-C^a) and 10 (B10.A(3R) × B10.A(5R))F_1 (Igh-C^b) mice as described (23–25). Individual bleeds were assessed for anti-Ia.17 activity by microcytotoxicity dye exclusion assays (25). Microcytotoxicity of serial dilutions of immune sera and a known concentration of 10-2.16 were compared to determine Ia.17-specific antibody concentrations.

Competitive inhibition binding RIA. To assess the specificity of anti-idiotypic antibodies for 10-2.16 and 10-3.6, a competitive inhibition binding assay was adopted from Pierce and Klinman (26). Polyvinyl chloride plates (Dynatech Laboratories Inc., Alexandria, VA) were incubated with 200 μl of rabbit anti-10-2.16 (1.5 μg/ml) for 6 to 8 hr at 22°C. Remaining binding sites were saturated with 1% BSA, 1% rabbit serum in PBS, 0.02% sodium azide (RIA buffer) for 1 hr. Then 100 μl of ^{125}I-10-2.16 or ^{125}I-10-3.6 (1 to 2 ng/15,000 cpm) were admixed with cold inhibitors and incubated for 14 to 16 hr at 22°C. Each step of the procedure was followed by extensive washing with PBS containing 0.02% sodium azide. The number of cpm per well was determined in a gamma counter and results are expressed as the percent of maximal bound uninhibited ^{125}I proband. Iodination was performed by the chloramine-T method (27).

The concentration of idiotype expressed in immune serum was assessed. The percent inhibition of ^{125}I-idiotype binding by a 1/100 dilution of immune serum to anti-idiotype was determined and compared to the concentration of cold idiotype that provided equivalent inhibition of ^{125}I-idiotype binding.

The ability of anti-idiotypic antiserum and various anti-Ia antibodies to inhibit 10-2.16 and 10-3.6 binding to Ia.17-positive cell targets was determined. Lipopolysaccharide (LPS) blasts were prepared from mouse spleen cells cultured for 3 days in RPMI containing 10% fetal calf serum, 2-mercaptoethanol (3×10^{-5} M) and 10 μg/ml LPS (Difco). The harvested cells were adhered to microtiter wells (2×10^5 blasts/well) with PBS/0.25% glutaraldehyde. After extensive washing and incubation with RIA buffer, the competitive inhibition binding RIA was performed as described.

Direct idiotype binding assay. Xenogeneic and allogeneic anti-10-2.16 antibodies were adsorbed to 10-2.16 coupled to Sepharose 4B and then radiolabeled. In this procedure, the radiolabeling of the antigen binding site is avoided (28). ^{125}I-anti-idiotypic antibodies were eluted with 0.1 M glycine-HCl, pH 2.2. In a direct binding assay, serial dilutions of anti-Ia antibodies were adhered to microtiter wells. After washing and incubation with RIA buffer, ^{125}I-anti-10-2.16 antibodies were incubated for 12 to 14 hr at 22°C. Results are expressed as cpm of ^{125}I-anti-10-2.16 bound. In addition, direct binding of radiolabeled 10-2.16 and 10-3.6 to anti-idiotypic reagents were also determined.

Biotin labeling of 10-3.6. Biotinyl-*N*-hydroxy-succinimide (BNHS) (Calbiochem, La Jolla, CA) was dissolved in dimethyl formamide at 1.7 mg/ml and an optimal amount of BNHS per milligram of protein was added. The reaction was performed in carbonate-bicarbonate buffer, pH 9.0, for 4 hr at 22°C and then dialyzed against PBS (29). The biotinylated Mab were then titrated for activity on positive and negative LPS blasts with fluoresceinated avidin (Becton Dickinson, Mountain View, CA) used as a developing agent.

Flow cytometric analysis. For flow cytometric analysis, LPS blasts were prepared as described. Red blood cells were removed by lysis with Tris-buffered ammonium chloride buffer. An appropriate dilution of biotinylated Mab was mixed with the cell suspension and incubated for 30 min. After washing away unbound antibody, 1 μg/50 μl fluoresceinated avidin was admixed with cells and incubated on ice for 30 min. Cells were fixed with 0.1% paraformaldehyde.

Samples were analyzed on an epics V flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a argon ion laser (coherent tuned to 488 nm). Forward angle light scatter was collected with an ND1 neutral density filter and 90-degree light scatter was collected unfiltered. Cellular fluorescence was collected in the range of 530 to 560 nm (emission range of fluorescein isothiocyanate). The flow cytometer was standardized with glutaraldehyde-fixed chicken erythrocytes with peak fluorescence emission brought to channel 100. Cells were analyzed at a rate of 100 per second and 10,000 cells were analyzed in each histogram. Fluorescence histograms were analyzed with the EASY data analysis systems (Coulter) consisting of a DEC 11/23 computer and TERAC 8086 processor.

RESULTS

Epitope specificity of 10-2.16 and 10-3.6. Both 10-3.6 and 10-2.16 recognize Ia determinants defined by the Ia.17 specificity, although it had not been determined whether these antibodies recognized the same or different epitope(s) (10). Competitive inhibition binding of ^{125}I-10-2.16 to Ia.17-positive LPS blasts was performed to identify the specificity of 10-2.16 and 10-3.6 Mab. The results of this competitive inhibition indicated that 10-3.6 could inhibit the binding of ^{125}I-10-2.16 to Ia.17-positive cell targets although not as effectively as 10-2.16 itself (Table II). Furthermore, the inhibition pattern of radiolabeled 10-2.16 by 10-3.6 was similar when cell targets from four different Ia.17-positive haplotypes were used. Thus, the two anti-Ia.17 Mab identify the same or overlapping epitope(s). The weaker inhibition of 10-2.16 binding by 10-3.6 may be due to the low avidity of 10-3.6 for the Ia.17 epitope(s) (30, 31).

Inhibition of 10-3.6 binding to Ia.17-positive cell targets by 10-2.16 confirmed the previous findings. Due to low avidity, ^{125}I-10-3.6 bound weakly to Ia.17-positive and Ia.17-negative cell targets (data not shown). Therefore, a biotin-avidin system was employed to increase

TABLE II

Inhibition of ^{125}I-10-2.16 binding to Ia.17-positive cell targets[a]

Inhibitors	B10.A $I\text{-}A^k$	B10.M $I\text{-}A^f$	B10.S $I\text{-}A^s$	PL/J $I\text{-}A^u$
10-2.16	1[b]	0.9	0.9	0.5
10-3.6	200	100	95	48
11-5.2	100 (0)[c]	100 (0)	100 (0)	100 (0)
14-4-4S	100 (0)	100 (0)	100 (0)	100 (0)

[a] LPS blasts were adhered to microtiter wells (2×10^5 blasts/well). After extensive washing and incubation with RIA buffer, 100 μl ^{125}I-10-2.16 were admixed with serial dilutions of cold inhibitors. The microtiter plates were incubated for 14 to 16 hr at 22°C and washed, and the number of cpm/well was determined in a gamma counter.

[b] Concentration of inhibitor (μg/ml) required to achieve 50% inhibition of ^{125}I-10-2.16 binding (I_{50}).

[c] When I_{50} was not attained, numbers in parentheses represent percent inhibition achieved with maximal concentration of inhibitor.

sensitivity of 10-3.6 binding. As shown in Figure 1, unlabeled 10-3.6 and 10-2.16 inhibited the binding of biotinylated 10-3.6 to Ia.17-positive cell targets as detected by flow cytometry. 10-2.16 inhibited biotinylated 10-3.6 binding to a greater degree than did 10-3.6. These findings confirmed the initial observation that 10-2.16 and 10-3.6 detect the same epitope(s) and that the avidity of 10-2.16 is greater than 10-3.6 for the shared Ia epitope.

Characterization and specificity of xenogeneic anti-idiotypic antibodies. Rabbit anti-10-2.16 serum obtained after secondary immunizations and absorbed on affinity columns was found to be specific for 10-2.16. As shown in Table III, this antiserum does not detect isotype or allotype specificities and had no reactivity with the MOPC-21 κ light chain expressed by the 10-2.16 hybridoma (10). In a competitive inhibition binding assay of ^{125}I-10-2.16 to rabbit anti-10-2.16, 90 ng/ml 10-2.16 inhibited 50% of radiolabeled 10-2.16 binding. 10-3.6 and an anti-Ia.2 Mab, 11-5.2, did not inhibit ^{125}I-10-2.16 binding to rabbit anti-10-2.16. From these results it could be concluded that the rabbit anti-10-2.16 antibodies detected an individual idiotype (IDI) expressed by 10-2.16.

These competitive inhibition binding assays, however, may detect only high affinity antibodies. To determine if low affinity antibody interactions were present, direct binding assays were performed. In a direct binding assay, ^{125}I rabbit anti-10-2.16 bound to as small an amount as 100 ng of 10-2.16 but failed to bind to 350 μg/ml of 10-3.6 (Fig. 2). ^{125}I-10-3.6 also failed to bind directly to rabbit anti-idiotype (data not shown). These results indicate that low affinity 10-3.6 idiotypic determinants were not identified and confirm that idiotype is not shared between the 10-2.16 and 10-3.6 as detected by the rabbit anti-10-2.16 antibodies.

Experiments were performed to determine the location of 10-2.16 idiotypic determinants identified by the rabbit anti-10-2.16 serum. As shown in Figure 3, rabbit anti-10-2.16 inhibited the binding of ^{125}I-10-2.16 to Ia.17-positive cell targets documenting that anti-idiotypic antibodies recognized determinants in or near the 10-2.16-combining site. To determine if rabbit anti-10-2.16 also recognized framework determinants, 10-2.16 was adhered to Ia.17-positive LPS blasts blocking the 10-2.16-combining site and leaving only framework idiotypic determinants exposed. As shown in Figure 4, the rabbit anti-idiotypic serum bound selectively to the previously adhered 10-2.16. These results suggest that rabbit anti-10-2.16 serum contains populations of antibodies with different specificities (i.e., for either the 10-2.16-combining site or for framework determinants).

Rabbit anti-10-3.6 serum was also prepared, and when

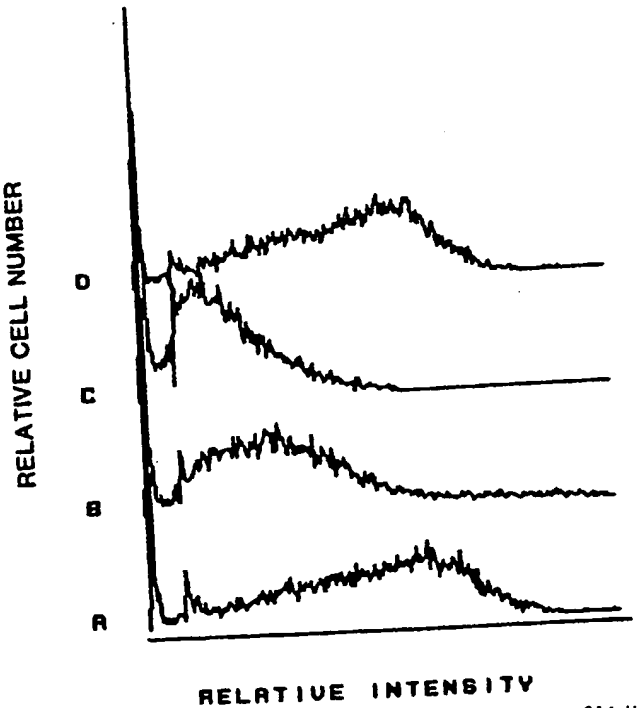

Figure 1. Flow cytometric profile of competitive inhibition of biotinylated 10-3.6 Mab binding to I-A^k LPS blasts. Various anti-Ia Mab were mixed with biotinylated 10-3.6 Mab (100 μg/ml). After 30 min incubation, cells were washed and developed with fluorescein-avidin conjugate. *A*, uninhibited; *B*, 10-3.6 Mab (1.0 mg/ml); *C*, 10-2.16 Mab (0.1 mg/ml); *D*, 11-5.2 Mab (1.2 mg/ml).

TABLE III

Inhibition of radiolabeled 10-2.16 and 10-3.6 to respective anti-idiotypic sera[a]

Inhibitors	Rabbit Anti-10-2.16 ^{125}I-10-2.16	C3H/J Anti-10-2.16 ^{125}I-10-2.16	Rabbit Anti-10-3.6 ^{125}I-10-3.6
10-2.16	90[b]	70	10,000 (20)
10-3.6	5,000 (2)[c]	5,000 (0)	30
MOPC-21	10,000 (12)	10,000 (5)	10,000 (20)
11-5.2	10,000 (10)	10,000 (10)	10,000 (35)
103.1C9	10,000 (0)	10,000 (0)	10,000 (32)
LPC-1	—	—	10,000 (20)
C57BL/6 immunoglobulin	10,000 (12)	10,000 (0)	10,000 (22)

[a] Microtiter wells were incubated with 200 μl anti-idiotypic sera for 6 to 8 hr at 22°C. After washing and incubation with RIA buffer, 100 μl ^{125}I-10-2.16 or ^{125}I-10-3.6 were admixed with serial dilutions of cold inhibitors and incubated for 14 to 16 hr at 22°C. Microtiter wells were washed and number of cpm/well was determined in a gamma counter.

[b] Concentration of inhibitor (ng/ml) required to inhibit 50% binding (I_{50}).

[c] When I_{50} was not attained, numbers in parentheses represent percent [illegible] maximal concentration of inhibitor.

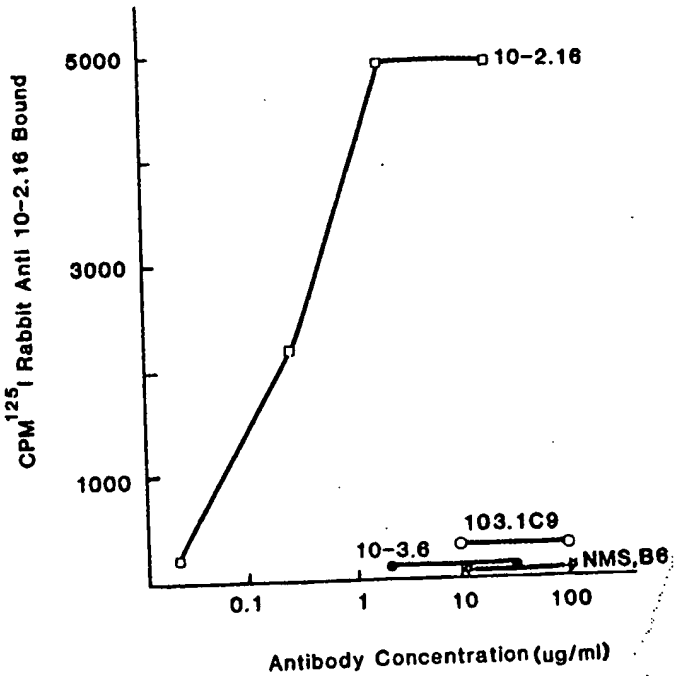

Figure 2. Direct binding assay of ^{125}I-rabbit anti-10-2.16 to variou Mab. Anti-idiotypic antibodies were radiolabeled while adhered to 1C 2.16 Mab Sepharose 4B column to prevent iodination of the idiotype specific combining site. Dilutions of Mab were initially adhered to micr titer wells and the assay was performed as described in *Materials an Methods*. NMS, normal mouse serum.

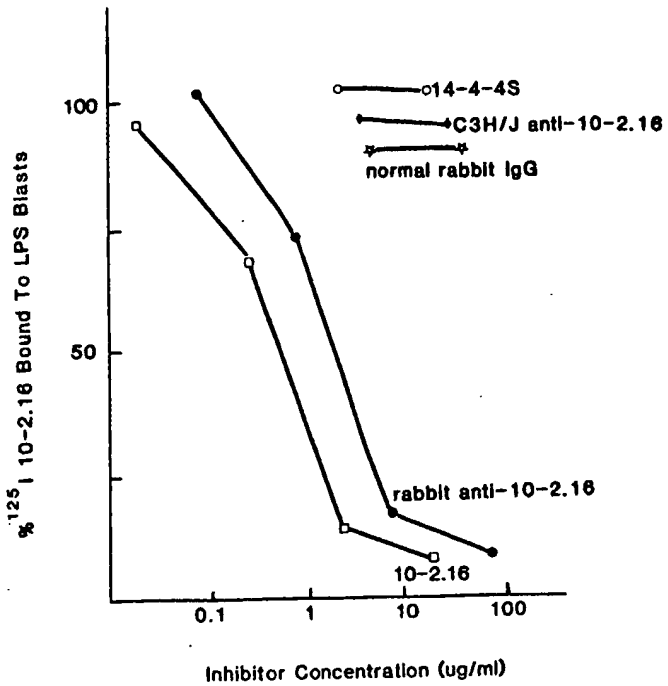

Figure 3. Inhibition of ^{125}I-10-2.16 binding to CBA/J (Ia.17-positive) LPS blasts by 10-2.16 Mab (□——□); rabbit anti-10-2.16 serum (●——●); C3H/J anti-10-2.16 serum (◆——◆); 14-4-4S Mab (○——○); and normal rabbit immunoglobulin (☆——☆).

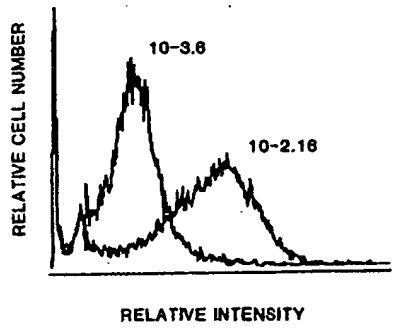

Figure 4. Rabbit anti-10-2.16 antibodies bind to 10-2.16 previously adhered to Ia.17-positive LPS blasts. Flow cytometric profile of rabbit anti-idiotypic antibodies bound to CBA/J LPS blasts that have been pretreated with 10-2.16 or 10-3.6 Mab. The rabbit antibodies are detected by fluoresceinated goat anti-rabbit immunoglobulin antibodies. Cytometric profile of cells pretreated with 10-3.6 Mab is similar to profiles obtained with normal rabbit immunoglobulin as the second antibody and the use of inappropriate C57BL/6 cell targets (data not shown).

tested, did not recognize isotype, allotype, or MOPC-21 κ light chain specificities (Table III). The binding of radiolabeled 10-3.6 to rabbit anti-10-3.6 was not inhibited by 10-2.16, and furthermore, ^{125}I-10-2.16 did not directly bind to this anti-idiotype (data not shown). These observations indicate that 10-3.6 and 10-2.16 do not share combining site-specific or framework-specific idiotype as detected by the two rabbit anti-idiotypic reagents.

Characterization of allogeneic anti-10-2.16 antibodies. C3H/J mice received weekly injections of 10-2.16/CFA i.p., and antibodies from the ascitic fluid obtained during the seventh week were found to be specific for 10-2.16. As shown in Table III, 70 ng/ml of 10-2.16 inhibited 50% of ^{125}I-10-2.16 binding to the C3H/J anti-10-2.16 serum. The C3H/J anti-10-2.16 serum did not detect allotypic specificites as demonstrated by the failure of C57BL/6 (Igh-C^b) immunoglobulins and 11-5.2 (Igh-C^b) to inhibit ^{125}I-10-2.16 binding. Similar to the findings with rabbit anti-10-2.16 serum, 10-3.6 did not inhibit ^{125}I-10-2.16 binding to the allogeneic anti-10-2.16 antibodies. Furthermore, low affinity antibodies to 10-3.6 idiotypic determinants could not be identified in that radiolabeled anti-idiotype did not directly bind to 10-3.6. Conversely, ^{125}I-10-3.6 did not bind directly to the C3H/J anti-10-2.16 serum (data not shown). These data confirm the findings observed with the xenogeneic anti-idiotypic serum, which demonstrate that idiotype is not shared between 10-2.16 and 10-3.6. Unlike the rabbit anti-10-2.16 serum, the C3H/J anti-idiotypic antibodies did not inhibit ^{125}I-10-2.16 from binding to I-A^k-positive LPS blasts (Fig. 3). Therefore, the allogeneic anti-idiotypic serum detects only 10-2.16 framework determinants.

10-2.16 and 10-3.6 idiotypes in anti-Ia.17 alloantisera. To determine the prevalence of the 10-2.16 and 10-3.6 idiotypes in immune sera, anti-Ia.17-containing alloantisera were obtained from 10 individual (B10.A(3R) × B10.A(5R))F$_1$ (Igh-C^b) mice and 10 BALB/c (Igh-C^a) mice immunized repeatedly with C3H/J spleen cells. Anti-Ia.17 activity of these sera was confirmed by cell cytotoxicity assay on I-A^k and I-A^f cell targets. The range of anti-Ia.17 antibody concentration for the immune sera was 0.5 to 15 μg/ml. Competitive inhibition binding assays revealed that anti-Ia.17 sera from three of 10 (B10.A(3R) × B10.A(5R))F$_1$ mice expressed 100 to 500 ng/ml of the 10-2.16 idiotypic determinants detected by the C3H/J anti-10-2.16 serum (Fig. 5*B*). This amount represented 6, 20, and 100% of the total anti-Ia.17 antibody responses for the respective 10-2.16 idiotype-positive immune sera.

Serum from each of the seven mice that failed to express the 10-2.16 idiotype was evaluated for the concentration of anti-Ia.17 antibodies. These experiments demonstrated that the anti-Ia.17 antibody concentrations of the three idiotype-positive immune sera did not differ from the anti-Ia.17 antibody concentrations of 10-2.16 idiotype-negative immune sera (data not shown). Thus, the failure to detect the 10-2.16 idiotype in these sera was not the result of low levels of anti-Ia.17 antibodies.

In contrast to the findings with the C3H/J anti-10-2.16 serum (Fig. 5*B*), the 10-2.16 and 10-3.6 idiotypic determinants detected by the rabbit anti-idiotypic sera were not identified in the (B10.A(3R) × B10.A(5R))F$_1$ anti-Ia.17 immune serum (Fig. 5*A* and *C*). In addition, 10-2.16 and 10-3.6 idiotypic determinants were not found in anti-Ia.17 sera from 10 BALB/c mice as shown by the failure of these sera to inhibit ^{125}I-10-2.16 and ^{125}I-10-3.6 binding to the three anti-idiotypic reagents (data not shown). Therefore, only 10-2.16 framework idiotypic determinants, detected by the allogeneic anti-10-2.16 serum, were found in anti-Ia.17 immune sera from Igh-C^b but not Igh-C^a mice.

DISCUSSION

In the present study, two Ia.17-specific Mab, 10-2.16 and 10-3.6, were found to recognize overlapping and possibly the same Ia epitope(s). Despite this observation, xenogeneic and allogeneic anti-idiotypic sera against these two anti-Ia Mab failed to detect a shared idiotype. The lack of a shared idiotype even though there is specificity for the same or overlapping Ia epitope(s) strongly suggests that the B cell response to specific Ia epitopes is more diverse than previously described (16–20). This conclusion was further supported by the variable expression of 10-2.16 idiotypic determinants in immune sera

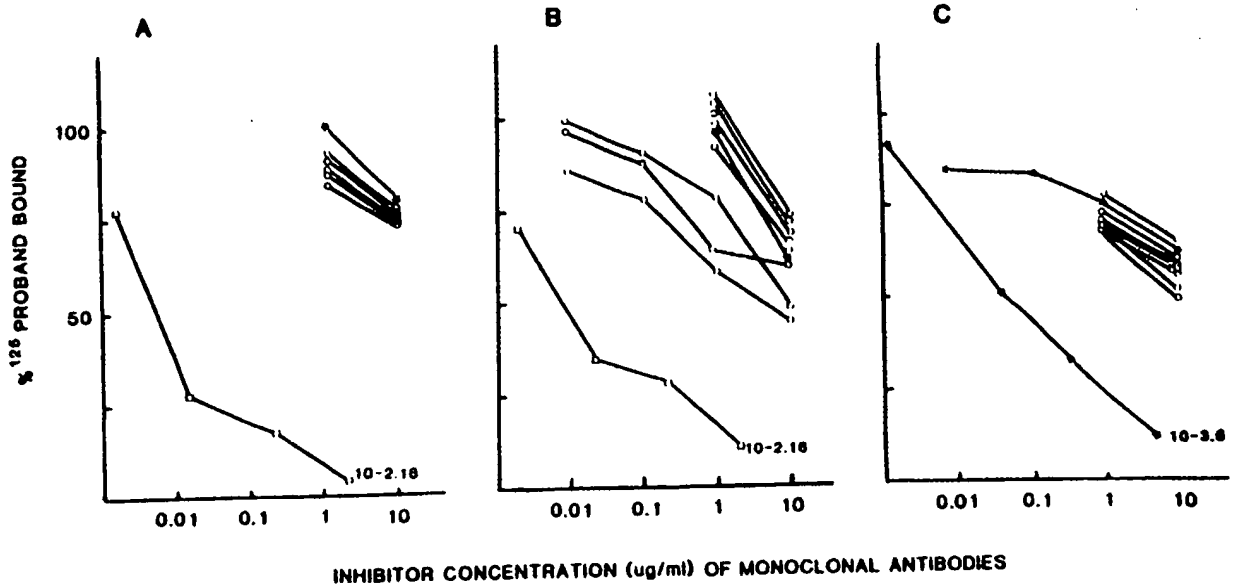

Figure 5. Inhibition by individual anti-Ia.17 immune sera from 10 (B10.A(3R) × B10.A(5R))F_1 mice of ^{125}I-10-2.16 or ^{125}I-10-3.6 binding to their respective anti-idiotypic reagents. *A*, ^{125}I-10-2.16 binding to rabbit anti-10-2.16 serum; *B*, ^{125}I-10-2.16 binding to C3H/J anti-10-2.16 serum; *C*, ^{125}I-10-3.6 binding to rabbit anti-10-3.6 serum. Inhibitor concentrations of individual anti-Ia.17 immune sera are serial 10-fold dilutions with an initial concentration of 1:10. Individual (B10.A(3R) × B10.A(5R))F_1 anti-C3H/J sera (O——O); normal mouse serum (☆——☆); 10-2.16 Mab (□——□); 10-3.6 Mab (★——★).

from mice immunized to Ia.17.

Although the results reported here indicate that the Ia.17-specific Mab 10-2.16 and 10-3.6 recognize a shared epitope, an alternative conclusion could be that they react with spatially related but distinct determinants. Our experiments and those of other investigators, however, argue against this latter interpretation. For example, other researchers have shown that the Ia.17 specificities expressed by the k, f, s, u, r, and j alleles were different, in that the dissociation rates of 10-2.16 and 10-3.6 bound to target cells from these haplotypes were different (30–32). Therefore, if 10-2.16 and 10-3.6 were detecting spatially related epitopes rather than a shared epitope, the competitive inhibition patterns of 10-2.16 binding to cell targets of different haplotypes should vary. This was not the case; the inhibition patterns of 10-2.16 binding to LPS blasts of k, f, s, and u by 10-3.6 were quite similar, indicating that they were reacting with a shared epitope.

As noted in the experiments reported above, 10-2.16 inhibited 10-3.6 binding to Ia.17-positive LPS blasts more effectively than 10-3.6 inhibited itself or the binding of 10-2.16. The higher dissociation rate of 10-3.6 reported by other investigators indicated that 10-2.16 has higher avidity than 10-3.6 and is consistent with the conclusion that 10-2.16 and 10-3.6 recognize a shared epitope (30, 31).

In addition to elucidating the fine specificity of Ia determinants, anti-Ia monoclonal probes have been employed to study the clonal diversity of anti-Ia immune responses. The idiotypy of anti-Ia antibodies appears to be limited in that several investigators have detected nondominant, combining site-specific CRI on many anti-Ia Mab. Mab to Ia.1, Ia.2, cluster I of Ia.7, and two I-A^b domains have been shown to express CRI (16–20). While expressing CRI, these Mab are clonally diverse in that they all express IDI. Conversely, some anti-I-E^k Mab that mutually inhibit each other's binding to Ia-positive cell targets do not share idiotype. It appears that these anti-I-E^k Mab do not recognize the same epitope(s), demonstrated by different patterns of cross-reactivity for H-2 cell targets (14, 18).

Whereas the 10-2.16 and 10-3.6 Mab reported here detect the same epitope, they do not appear to share an idiotype. The xenogeneic anti-10-2.16 serum used in our experiments contained antibodies specific for the 10-2.16-combining site and framework-specific idiotypes. Direct and indirect binding assays revealed that the 10-2.16 idiotype detected by this anti-idiotype serum was not shared by 10-3.6. Conversely, the 10-3.6 idiotype detected by xenogeneic anti-10-3.6 antibodies was not shared by 10-2.16. Although the idiotypic repetoire to many Ia specificities is limited, our data indicate that the B cell response to the Ia.17 specificity is diverse. This conclusion was further supported by the heterogeneous expression of the 10-2.16 idiotype in anti-Ia.17 immune sera.

10-2.16 idiotypic determinants detected by C3H/J anti-10-2.16 sera were found in three of 10 Igh-C^b mice expressing anti-Ia.17 activity. The antibody concentration of antibodies expressing the 10-2.16 idiotype represented 6, 20, and 100% of the total anti-Ia.17 antibody response for these three mice. This variable expression of 10-2.16 idiotype-containing antibodies further supported the diversity of the clonal response to the Ia epitope detected by 10-2.16 and 10-3.6. The expression of 10-2.16 framework-specific idiotype in immune sera also indicated that the Mab 10-2.16 was not a somatic mutation or an artifact of hybridization. This conclusion was further supported by the unresponsiveness of C3H/J mice to the 10-2.16-combining site-specific idiotype. Because 10-2.16 was isolated from a CWB anti-C3H/J immunization, failure to detect 10-2.16-combining site-specific idiotype indicated that these determinants may be expressed by syngeneic (C3H/J) B or T cell receptors.

CRI expressed by anti-Ia Mab is generally found in Igh-C identical immune sera, although the Ia.1 and Ia.7 cluster I-specific idiotypes are expressed by several allotypes (19). CWB, from which 10-2.16 and 10-3.6 were isolated,

is of C3H.SW background, but expresses the Igh-C^b allotype similar to C57BL/10 (33). Recent evidence suggests that CWB V_H genes distal to the NP^b idiotypic marker may not express the C57BL/10 allotype (34, 35). The 10-2.16 idiotype was found in Igh-C^b but not Igh-C^a anti-Ia.17 immune sera. This finding indicates this idiotype is encoded in the C57BL/10 V_H gene. Studies are in progress to clarify this finding further.

Molecular genetic analysis has identified a limited number of Ia molecules encoded within the *I* region (36). Mechanisms are needed, therefore, to explain how the small number of Ia molecules restricts the immune responses to numerous antigens. One explanation is that I-A and I-E molecules express multiple epitopes and it is the individual epitopes on each Ia molecule that restrict immune responses to specific antigens (37, 38). Our data suggest an additional mechanism for creating diversity of *Ir* gene-restricted immune responses. We observed that idiotype was not shared between two anti-Ia Mab specific for the same epitope. Therefore, heterogeneous B cell receptors specific for the same Ia epitope may provide an additional level of diversity for *Ir* gene-restricted immune responses.

Furthermore, evidence continues to accumulate supporting the idiotype interaction of B and T cells in humoral and cellular responses (39, 40). In particular, idiotype-specific helper T cells for an anti-H-2 antibody response have been identified (41). These findings, along with our data, support the role of Ia antigen-specific B and T cell idiotypy in Ia antigen-mediated control of immune responses.

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Generation of monoclonal antibodies to the zinc finger domain of the eukaryotic transcription factor Sp1

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Abstract

Using a synthetic peptide that encompasses the zinc finger domain of the eukaryotic transcription factor Sp1, we produced a number of monoclonal antibodies that specifically reacted with the target antigen. Analysis by competitive inhibition assay of five of the monoclonal antibodies revealed that they all recognized a dominant epitope in the synthetic peptide and reacted strongly to recombinantly synthesized β-galactosidase-Sp1 fusion polypeptide. To determine cellular distribution of Sp1-like molecules, cytoplasmic and nuclear proteins from human lung fibroblasts (HFL-1) and a human rhabdomyosarcoma cell line (A204) were immunoblotted and reacted with our antibodies. In addition to the well characterized 95 Kd and 105 Kd proteins, considered to be the authentic Sp1 polypeptide, a number of other cellular proteins reacted with these antibodies. Immunofluorescence staining of the cells with mAb to the zinc finger of Sp1 also revealed cell-specific differences in intracellular distribution of Sp1-like molecules. Both cytoplasmic and nuclear staining was readily observed in the rhabdomyosarcoma cells. In contrast, while some HFL-1 cells exhibited staining of only cytoplasm, both cytoplasmic and nuclear immunofluorescence was seen in others.

Introduction

Transcriptional regulation of genes in eukaryotes is a complex process, precise mechanisms of which are not well characterized. For genes transcribed by RNA polymerase II, specific interaction between *cis*-regulatory elements of DNA and transcriptional factors that recognize these sequences is an important prerequisite to assembling an actively-transcribing complex [1–3]. A number of these transcriptional regulatory factors, both tissue-specific and ubiquitous, have been described. Sp1 is one of the ubiquitous transcription factors known to activate cellular and viral promoters *in vitro* [4–7]. Activity of Sp1 was first detected in HeLa cells by virtue of its ability to selectively activate transcription from the SV40 early promoter. Sp1 has been shown to bind to a GC rich decanucleotide sequence known as the GC box [8–11]. By using a synthetic homolog of GC box covalently bound to cellulose, Sp1 was purified to apparent homogeneity by affinity chromatography [12, 13]. Complete primary sequence of Sp1 has been deduced from its cloned cDNA [14]. The DNA binding activity of recombinantly synthesized Sp1 was located in the COOH-terminal 168 amino acid residues of the polypeptide. In this region Sp1 contains three contiguous zinc finger motifs which are believed to be metalloprotein structures involved in sequence-specific binding to DNA [15]. In addition to the DNA binding domain, Sp1 contains multiple domains involved in transcriptional activation; the two most potent of these domains are glutamine-rich stretches located in the NH_2-termi-

nal half of the protein [16, 17]. Study of the DNA binding and transcriptional activation domains of transcription factors has been greatly facilitated by "domain-swapping" experiments [1–3]. Additional information has been gathered by co-transfection of plasmids containing genetically altered transcription factors and test promoters known to be activated by these. A consensus that emerges from these studies is that several transcriptional factors can bind to the same target DNA. Thus, clear dissection of both protein-DNA and protein-protein interactions will be essential to understanding the process of transcriptional activation. A potentially powerful approach to investigating the role and mechanisms of transcription factors *in vivo* and *in vitro* may be to develop and utilize domain-specific monoclonal antibodies to decipher various molecular interactions. Towards a long term objective of utilizing such reagents in our studies on transcriptional regulation, we report here our successful generation of monoclonal antibodies by using a synthetic peptide representing the zinc finger domain of transcription factor Sp1 as the antigen. These monoclonal antibodies recognize both bacterially-produced Sp1 as well as Sp1-like molecules in two mesenchymal cell lines.

Material and methods

Cell culture

BALB/c mouse myeloma cell line Sp2/0-Ag14 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS), 1 mM glutamine and 100 units/ml of penicillin and 100 μg/ml streptomycin. A line of human lung fibroblasts (HFL-1) obtained from American Type Cell Culture was also cultivated as monolayers in DMEM with 10% FCS. A204 cells, derived from a human rhabdomyosarcoma were obtained from Dr. Helene Sage (University of Washington, Seattle, Washington) and were also grown in DMEM with 10% FCS.

Preparation of monoclonal antibodies against the zinc finger domains of transcription factor Sp1

Monoclonal antibodies against the zinc finger domain of the human Sp1 were prepared according to previously published methods [18, 19]. A peptide corresponding to the amino acid residues 520–619 of the human Sp1 protein, containing the presumptive DNA binding zinc fingers, was synthesized in an automated peptide synthesizer (Applied Biosystems Inc., CA). Synthetic peptide was solubilized in distilled water (4 mg/ml) and emulsified with an equal volume of Freund's complete adjuvant. Emulsified antigen (100 μl/mouse) was injected subcutaneously into BALB/c mice; the secondary immunizations with 0.5 ml solution of synthetic Sp1 (200 μg/ml in distilled water) were done on day 13 after the first injection. On the third day after second immunization, immune spleen cells were collected and fused with BALB/c Sp2/0-Ag14 myeloma cells using 45% polyethyleneglycol according to the method of Kohler and Milstein [20]. Hybridomas were selected in DMEM containing 100 μM hypoxanthine, 0.4 μM aminopterine, and 10 μM thymidine. The established hybridoma cultures were cloned by limiting dilution. Individual hybridomas were expanded and culture supernatants were assayed for their antibody titer against the synthetic Sp1 as antigen by enzyme-linked immunosorbent assay (ELISA).

Antibody purification

Hybridomas were cultured in DMEM and IgG in the culture supernatant was purified by affinity chromatography using synthetic Sp1 coupled to Actigel A (Sterogene Biochemicals), San Gabriel, CA); bound IgG was eluted with 0.1 M glycine-HCl, pH 3.0. The purified antibodies were dialyzed extensively against phosphate buffered saline, pH 7.2 (PBS) and filter-sterilized. Concentration of IgG was determined spectrophotometrically by reading the absorbance at 280 nm.

Enzyme-linked immunosorbent assay (ELISA)

Micro-ELISA plates (COSTAR, Cambridge, MA) were coated overnight at 4° C with 100 μl/well of 0.1 M carbonate buffer (pH 9.6) containing 5 μg/ml of the synthetic Sp1. To remove the unbound antigen, wells were washed with Tris-buffered saline, pH 8.0 containing 0.05% Tween-20 (TBS-Tween). Following washing, wells were incubated with 50 μl

of TBS-Tween and 50 μl of either hybridoma culture supernatant or purified IgG at room temperature for 2 hr. Wells were then incubated for an additional 2 hr at room temperature with 100 μl of peroxidase-conjugated goat anti-mouse IgG (TAGO, Burlingame, CA) diluted 1: 2000 in TBS-Tween and excess antibody was removed by washing with TBS-Tween. Peroxidase reaction was initiated by incubation of wells with 100 μl of 0.06% H_2O_2 in citrate phosphate buffer, pH 5.2 containing 0.5 mg/ml O-phenylenediamine for 30 min at room temperature; enzymatic reaction was stopped by addition of 50 μl of 2.5 N H_2SO_4. The titers of antibodies were estimated by measuring the absorbance at 490 nm/650 nm with an ELISA reader (MOLECULAR DEVICES, Palo Alto, CA).

Biotinylation of monoclonal antibody and competitive inhibition assay

To determine the epitope specificity of anti-Sp1 antibodies, we used the biotinavidin system and a competitive inhibitions assay. Purified monoclonal antibodies were coupled to biotin. Monoclonal antibodies (1 mg/ml) were dialyzed against 0.01 M $NaHCO_3$ at 4° C overnight. One hundred microliter of N-hydroxysuccinimide biotin in DMSO (1 mg/ml) was added to 1 ml of monoclonal antibody solution; the mixture was incubated for 4 hr at room temperature and dialyzed against PBS with 0.1% NaN_3.

Micro-ELISA plates were coated with 100 μl of a solution of the synthetic Sp1 (5 μg/ml) and excess unbound antigen was removed by washing with TBS-Tween; serial dilution of the unlabeled antibody to be tested were then added to each well (50 μl/well). Fifty microliters of purified biotinylated antibody in TBS-Tween containing 0.5% ovalbumin was added and after 2 hr incubation at room temperature, wells were washed extensively with TBS-Tween. After the addition of 100 μl of avidin coupled to peroxidase 1: 500 in TBS-Tween containing 0.5% ovalbumin, binding of the biotinylated antibody was determined. The percent inhibition of antigen-antibody binding by the competitor was calculated using the following formula:

$$\% \text{ inhibition} = 1 - \frac{\text{Absorbance with inhibitor}}{\text{Absorbance without inhibitor}} \times 100$$

Immunoblot analysis

To check specificity of monoclonal antibody, crude lysate of *E. coli* strain JM83 containing pLacZ-Sp1-168C was prepared according to the method of Kadonaga *et al.* [14]. In order to reduce proteolytic degradation of the fusion proteins, in some experiments *E. coli* strain Y 1090, lacking *lon* protease was used to express the β-gal-Sp1 fusion protein. Plasmid pLacZ-Sp1-168C was obtained from Dr. Robert Tjian, Department of Biochemistry, University of California, Berkeley, California; it encodes the entire β-galactosidase (116 Kd) fused to the COOH-terminal 168 amino acid residues (18 Kd) of Sp1. Nuclear extracts and cytosolic S100 fractions were prepared from A204 or HFL-1 cells as described by Dignam *et al.* [21]. Polypeptides in crude bacterial lysate, cell or nuclear extracts were size-fractionated by SDS polyacrylamide gel electrophoresis [22]. Gels were soaked in transblotting buffer (25 mM Tris, 200 mM glycine, 20% methanol pH 8.3) for 20 minutes and proteins were electroblotted onto nitrocellulose (14V constant voltage overnight at 4° C) in transblotting buffer. To circumvent nonspecific protein binding, filters were incubated in TBS containing 2% normal goat serum (NGS) for 1 hr at room temperature on a rocking platform. As a source of primary antibody, hybridoma culture supernatants were diluted 1 : 10 in blocking buffer. Second antibody was peroxidase conjugated goat anti-mouse IgG and was diluted 1 : 1000 in blocking buffer. Incubations with primary antibody were overnight at 4° C and incubations with second antibody were for 2 hr at room temperature. Peroxidase activity was visualized using 0.5 mg/ml 4-chloro-1-naphtol as substrate.

Immunofluorescence

Cells were grown on 18 mm glass cover slips in DMEM with 10% FCS. Subconfluent monolayers of cells were fixed (3.7% formaldehyde in PBS) for

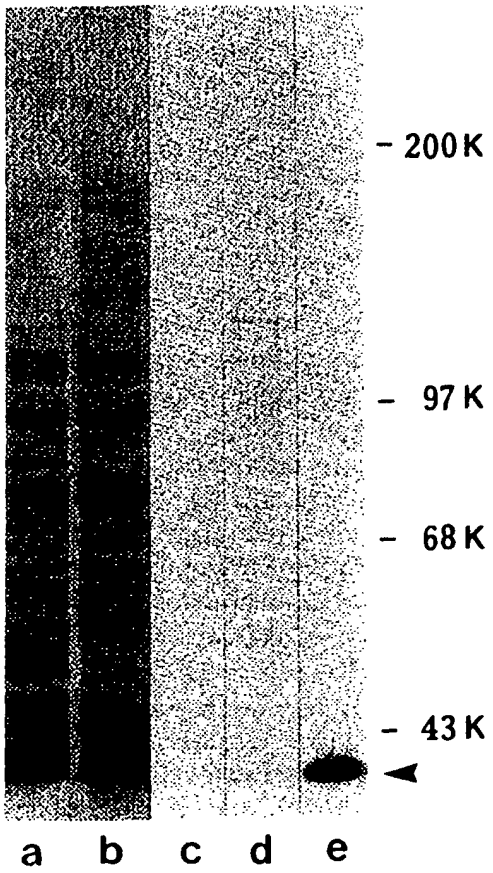

Fig. 1. Immunoblot detection of Sp1-168C with mAb S42. Crude protein lysates prepared from *E. coli* stain JM83 without (a) or with (b) pLacZ-168C (100 μg/lane) were electrophoresed through 6% SDS-PAGE and stained with coomassie brilliant blue. Polypeptides of lysates from *E. coli* were transblotted and reacted with mAb S42. While no detectable binding of mAb S42 is seen in JM83 extracts without pLacZ-168C (c), two major and one minor polypeptide bands react in crude lysates of *E. coli* containing pLacZ-Sp1-168C (d). Synthetic Sp1 peptide is readily recognized by mAb S42 (lane e, arrow). Location of molecular weight markers electrophoresed in a parallel lane has been marked.

20 min at room temperature and permeabilized with 100% acetone at −20° C for 5 min. After extensive washing with PBS, each cover slip was incubated with undiluted hybridoma supernatant containing 2% NGS overnight at 4° C. Following specific antibody antigen interaction, cover slips were washed with PBS containing 2% NGS; this was followed by incubation in 1 : 20 diluted fluorescence conjugated goat antimouse IgG overnight at 4° C and extensive washing in PBS containing 2% NGS. The cells were then mounted in a solution of p-phenylenediamine at 1 mg/ml in 90% glycerol, pH 8.0 [23] and viewed under Zeiss photomicroscope Axiophot. Photographs were taken using Kodak Tri-Xpan film at ASA 400.

Results

Specificity of monoclonal antibodies

Initially, hybridoma supernatants were tested against COOH-terminal synthetic fragment of Sp1 bound to microtiter plates; these monoclonal antibodies also reacted strongly with the synthetic peptide in an immunoblot (see below). Finally, to test if recombinantly produced fragment of Sp1 in *E. coli* was also readily recognized by these monoclonal antibodies, we reacted total cytoplasmic extracts prepared from *E. coli* containing pLacZ-Sp1-168C, which express the β-galactosidase fused to the COOH-terminal 168 amino acids of the Sp1. The results of immunoblot analysis are shown in Fig. 1. Monoclonal antibody S42 reacted with three polypeptide species in the bacterial lysate with apparent molecular mass of 130, 110, 90 Kd, two of which (130 Kd and 110 Kd) accounted for majority of the immunoreactivity. We believe that the 130 Kd polypeptide represents the authentic β-gal-Sp1 fusion protein, while the 90 and 110 Kd polypeptide bands are most likely products of proteolytic degradation of β-gal-Sp1 fusion polypeptide. The notion of 90 and 110 Kd polypeptides being the truncated forms of β-gal-Sp1 fusion protein is further supported by our analysis of the recombinant proteins made in the strain Y 1090 of *E. coli*. When expressed in Y 1090, which lacks *lon* protease, only the 130 Kd polypeptide is detected (unpublished observation). It is well known that the β-gal-fusion polypeptides are much more stable in strains of *E. coli* lacking *lon* protease [24]. None of the polypeptides found in the crude lysate of *E. coli* strain JM83, which did not contain Sp1 expressing plasmid, reacted with the putative zinc finger reactive monoclonal antibodies (Fig. 1).

Epitope specificity of monoclonal antibodies

From a total of four fusions with spleen cells isolat-

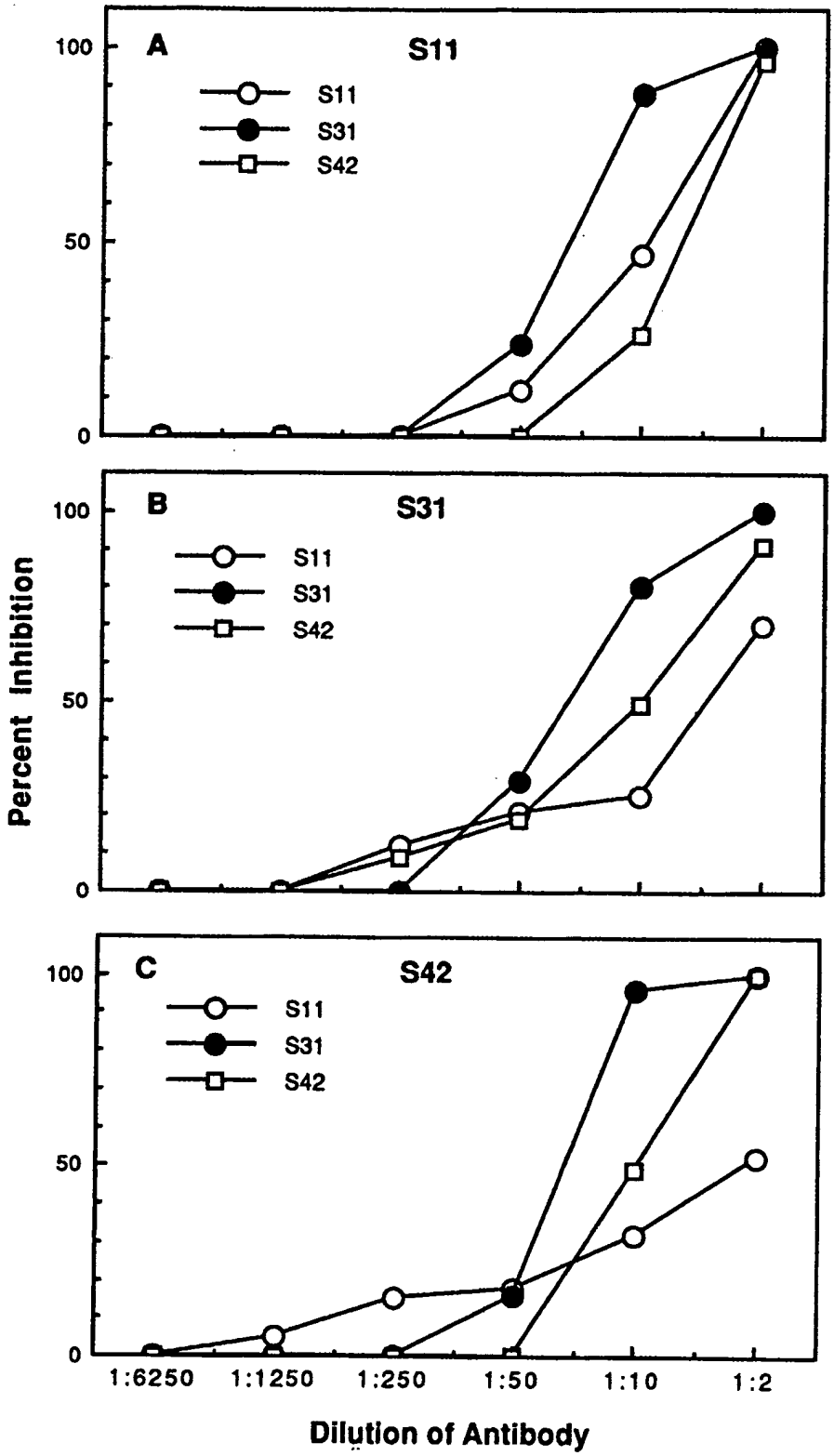

Fig. 2. Competitive inhibition of binding of biotinylated monoclonal antibodies to synthetic Sp1. Micro-ELISA plates were coated with synthetic Sp1 and reacted with biotin-labeled monoclonal antibody in the presence of either homologous or heterologous mAbs at dilutions indicated in the x axis. Percent inhibition of binding to a specified antibody was determined as described in detail in the Materials and methods.

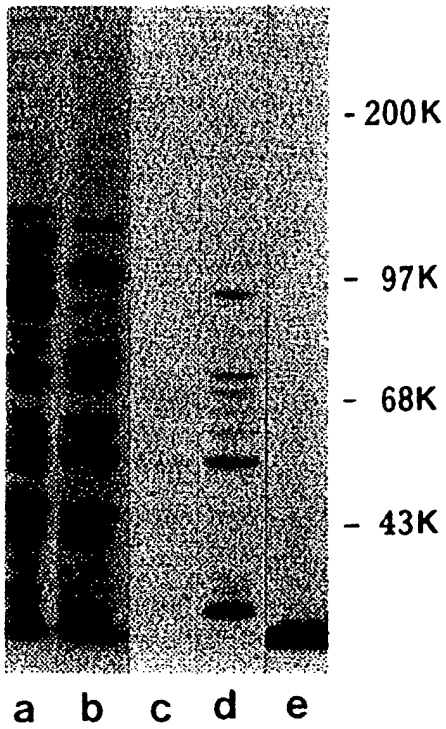

Fig. 3. Immunoblot detection of Sp1 with mAb S42 in A204 cell extract. Cytoplasmic (a) or nuclear fractions (b) from A204 cells (100 μg protein from each fraction) were size-fractionated in 7.5% SDS-PAGE and stained by coomassie brilliant blue (a, b). Polypeptides were immuno-blotted and reacted with affinity-purified mAb S42; both cytoplasmic (c) and nuclear (d) fractions showed reactivity. Lane e contained 1 μg of synthetic Sp1 peptide which reacted strongly (arrow). Migration of molecular weight markers run in a parallel lane is also depicted.

ed from BALB/c mice immunized with the synthetic Sp1, we obtained several hybridomas that strongly reacted with the synthetic peptide. We selected five of these monoclonal antibodies to examine their epitope specificity by competitive inhibition assay performed by using biotin labelled antibodies. The results with three monoclonal antibodies are shown in Fig. 2. The binding of each of the three biotinylated antibodies to the synthetic Sp1 was inhibited by culture supernatant representing homologous and heterologous antibodies in a dose dependent manner. The results depicted in Fig. 2 were similarly mimicked by the remaining monoclonal antibodies (data not shown). Based on these data, we conclude that all of these antibodies recognized a dominant epitope. Conceivably, either all these monoclonal antibodies recognized the same epitope or different epitopes in close physical proximity of each other.

Immunoblot analysis

Nuclear and cytosolic fractions were prepared from A204 or HFL-1 cells as described in Experimental Procedures and analyzed by immunoblotting techniques. Results for A204 cells are presented in Fig. 3 which displays the coomassie blue stained polypeptide profile and corresponding nitrocellulose blot probed with mAb S42. mAb S42 recognized 95 Kd and 105 Kd bands; similar molecular weight polypeptides were identified in HeLa cell extracts previously as authentic Sp1 species. 95 Kd and 55 Kd bands were clearly the major species in nuclear extract reacting with mAb S42. Additional minor bands were, nevertheless, present in nuclear extract. We have observed that the lower molecular weight species were variably present and are most likely produced by proteolytic degradation of Sp1. Another minor band of approximate molecular mass of 200 Kd could represent aggregation of Sp1. Alternatively, the minor species may represent Sp1 bearing variable amounts of O-linked N-acetylglucosamine residues [25]. However, in the absence of direct experimental evidence, this is speculative and the minor polypeptides may actually be other proteins containing zinc fingers which are recognized by our monoclonal antibody. Surprisingly, neither 95 Kd nor 105 Kd band was a major species in the cytoplasmic fraction (Fig. 4). Immunoblot profile of polypeptides representing nuclear and cytoplasmic fractions from lung fibroblasts essentially resembled that obtained from A204 cells (results not shown).

Immunofluorescence microscopy

In order to detect intracellular distribution of Sp1-like molecules, immunofluorescence was performed using A204 or HFL-1 cells fixed with 3.7% formaldehyde and permeabilized with acetone. Immunofluorescence labeling of A204 cells by mAb S42 is shown in Figure 4C. MAb S42 produced in a finely reticular pattern of immunofluorescence throughout the cytoplasm and nucleus; conspicuously, mAb S42 did not stain the nucleoli. In -

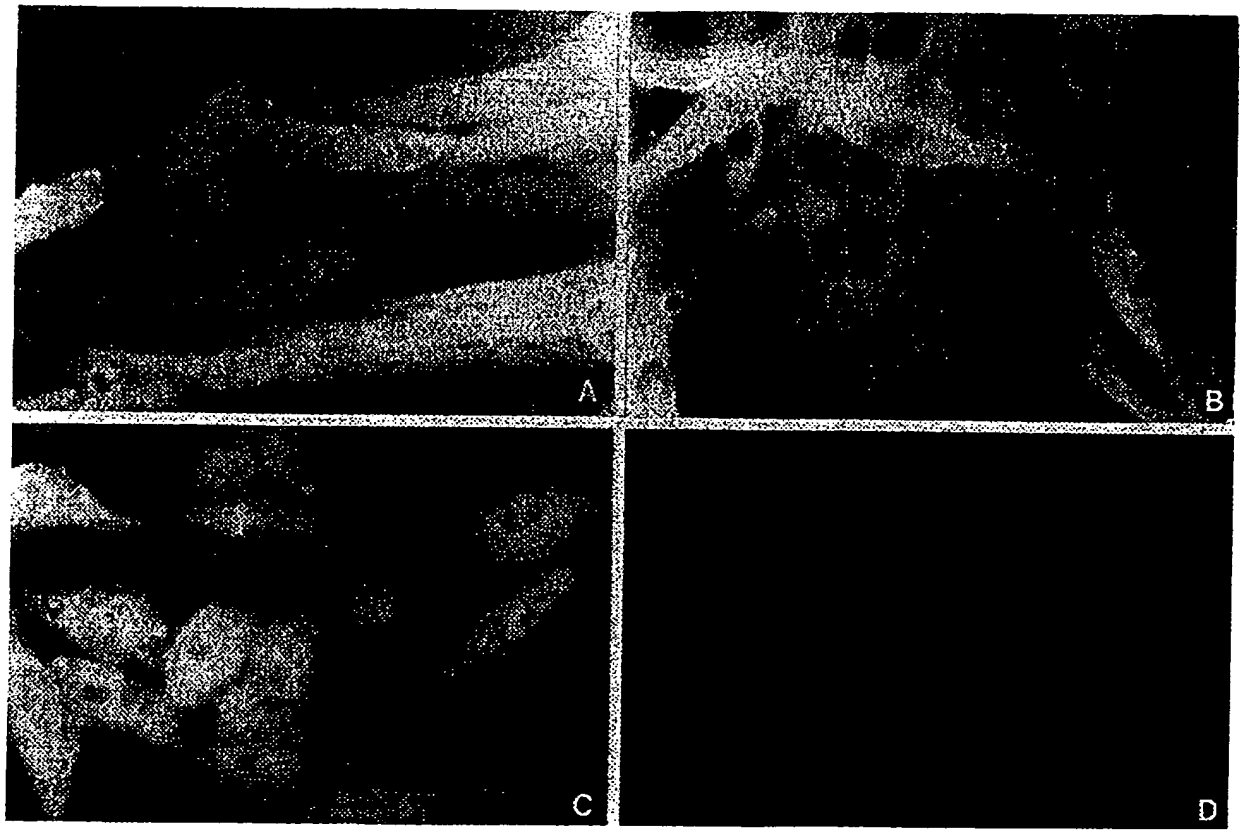

Fig. 4. Distribution of Sp1 zinc finger-containing polypeptides in HFL-1 and A204 cells. Cells grown on glass cover slips were fixed, permeabilized and reacted with affinity-purified mAb S42. Antibody antigen-interaction was detected by fluorescence-conjugated goat anti-mouse IgG as described in the Materials and methods. HFL-1 cells (A, B) and A204 cells (C, D) were reacted with either mAb S42 (A, B, C) or with an antibody reactive with type II collagen (D). Bar in C represents 10 micrometers.

contrast, HFL-1 cells exhibited two types of staining. Some cells showed reticular staining of nucleus and cytoplasm similar to that observed in A204 cells (Fig. 4A), while in other cases, staining was only located in the cytoplasm (Fig. 4B). In order to test the background immunofluorescence, we used monoclonal antibody against type II collagen as the primary antibody (an irrelevant mAb, since none of these cells synthesize type II collagen). There was minimal background fluorescence as shown in Fig. 4D.

Discussion

Using a synthetic peptide representing the COOH-terminal zinc finger domain of Sp1, we successfully generated several monoclonal antibodies against Sp1. We have extensively tested five of these mAbs, named S11, S24, S31, S42 and S73. According to the results of competitive inhibition study, all five of these mAbs recognized same or juxtaposed epitopes suggesting that there is a dominant epitope that is strongly antigenic. Using mAb S42, which apparently had the highest affinity for Sp1, we tested various parameters of specificity. We used crude lysate of *E. coli* strain JM83 harboring pLacZ-Sp1-168C which encodes the entirety of β-galactosidase (116 Kd) fused to the COOH-terminal 168 amino acid residues (18 Kd) of Sp1 [14]. Our antibody recognized 130 Kd and 110 Kd polypeptide bands in the extract of *E. coli* strain JM83 containing pLacZ-Sp1-168C. In the *E. coli* strain Y 1090, the 130 Kd polypeptide is the only species -

detectable with our mAb. Thus, based on the expression of β-gal-Sp1 fusion polypeptide in a *lon*⁻ strain we believe that the lower molar weight species are proteolytically truncated fragments of the fusion protein.

In addition to the 95 and 105 Kd polypeptides considered to be different posttranslationally modified forms of the authentic Sp1 [14], we detected a number of other polypeptide species in both A204 and HFL-1 cells. This is consistent with previously published findings which showed that partially purified Sp1 contained several polypeptides all of which displayed Sp1 activity; Briggs *et al.* [13] observed minor species at 190, 115 and 110 Kd and trace amount of polypeptides smaller than 90 Kd. Some of the polypeptide species we observed in A204 and HFl-1 cells corresponded in their molecular mass to those previously observed [13]. Alternatively, it is known that Sp1 is subject to multiple posttranslational modifications [25]. Whether some of the minor (or major) polypeptides recognized by our mAbs represent products of proteolysis or posttranslational modifications of Sp1 is currently unknown.

Tissue-specific variation in the distribution of the various molecular subspecies of Sp1 should also be considered. For example, Sp1 purified human placenta was shown to be a polypeptide of approximately 40 Kd rather than 105 or 95 Kd species found to exist in HeLa cells. It is conceivable, therefore, that the 55 Kd species, which was the predominant form of Sp1 in A204 cells, represented a genuine functional subspecies of the authentic Sp1. It is also conceivable that some of the molecular species of Sp1 may be generated by alternative splicing of the cognate mRNA encoding the protein. Whether generation of alternatively-spliced forms of Sp1 actually occurs in a tissue specific manner is not yet known. Finally, there is a less interesting possibility that in addition to the authentic Sp1, our mAbs cross-reacted with other proteins containing zinc finger domain. A precedent for such cross-reactivity was shown recently; a mAb to BGP1, an erythrocyte-specific protein that binds to poly(dG) region of chicken β-globin gene, was shown to recognize Sp1 [26].

The patterns of immunoreactivity of Sp1-specific mAbs in intact cells resembled those observed previously in studies of the intracellular localization of other transcription factors [27, 28]. Transcription factor v-jun was localized in the nucleus with prominent lack of nucleolar staining [28]; this is similar to what we have observed in staining of two different cell types with mAbs to Sp1 zinc finger domains. Although nuclear localization of Sp1 is consistent with the site of action of this transcription factor, it is possible that our mAb recognized either precursor or degradation product(s) which may be abundant in cytoplasm at a particular stage in the cell cycle. On the other hand, Sp1 may shuttle back and forth between nuclear and cytoplasmic compartments. Subcellular distribution of Sp1-like molecules is of particular interest, since a preferential compartmentalization of transcription factors may be an important mechanism of control of transcriptional activation [29].

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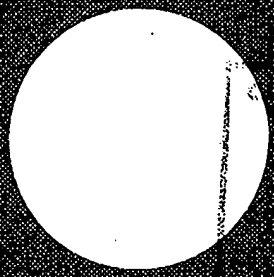

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